GENE ENCODING SYNTAXIN INTERACTING PROTEIN

BACKGROUND OF THE INVENTION

The present invention relates to novel genes and polypeptides derived therefrom encoding a syntaxin interacting protein. The invention also describes vectors and host cells comprising the novel gene. The invention further describes methods for using the novel gene, polypeptides, and antibodies specifically targeting the polypeptides, in the detection of genetic deletions of the gene, subcellular localization of the polypeptide, isolation of discrete classes of RNA, gene therapy applications, diagnostics for syndromes involving abnormal levels of glucose or abnormal GLUT4 translocation, development of proprietary screening strategies for inhibitors of syntaxin interacting protein.

SUMMARY OF THE RELATED ART

Insulin stimulation of glucose transport in the major insulin responsive cell types, muscle, skeletal and fat, occurs by the recruitment of glucose transporters, in particular GLUT4, from the intracellular low density microsomal compartment to the cell surface. A certain class of proteins have been implicated in the insulin-induced translocation of GLUT4 to the plasma membrane. This class of proteins have been referred to as SNARE proteins.

SNARE proteins are vesicle membrane and target membrane soluble N-ethylmaleidide-sensitive factor attachments protein receptors. SNARE proteins identified in the vesicle membrane, or v-SNAREs, are synaptobrevin or VAMP. SNARE proteins identified in the target membrane, or t-SNAREs, are syntaxin and SNAP-25.

Recent studies have demonstrated that isoforms of syntaxin, namely syntaxin-4, and VAMP, namely VAMP2 and VAMP3/cellubrevin, are required functional t-SNAREs and v-SNAREs for the insulin-stimulated GLUT4 translocation to the plasma membrane. GLUT4 translocation plays an important role in the uptake of glucose by cells, which in turn plays an important role in

disease states characterized by abnormal glucose uptake. By gaining an understanding of the biochemical mechanisms behind these required v- and t-SNAREs and their effect on insulin-stimulated GLUT4 translocation, new opportunities for treating and diagnosing diseases related to abnormal (high or low) storage and/or utilization of glucose, may be achieved. Stated another way, a better understanding of the molecular mechanisms of glucose transport will allow improved design of therapeutic drugs that treat diseases related to abnormal storage and/or utilization of glucose. Such disease states include diabetes, glycogen storage diseases, obesity, polycystic ovarian syndrome, hypertension, atherosclerosis and other diseases of insulin-resistance.

SUMMARY OF THE INVENTION

The invention relates to the discovery and purification of a novel target membrane protein (SNARE) syntaxin-4 interacting protein ("SYNIP") and the isolation of polynucleotide sequences encoding the proteins. SYNIPs are of interest because they may play an important role in the translocation of GLUT4 from the intracellular compartment to the cell surface in response to the presence of insulin. SYNIPs competitively bind to syntaxin-4 and prevent the ligand from interacting with its cognate intracellular receptor. This property of SYNIPs has profound physiological effects. Thus, by regulating the intracellular levels of the subject SYNIPs, desirable physiological effects may be obtained. Such effects may be used to treat a variety of diseases involving abnormal levels of glucose or the abnormal translocation of GLUT4 (ie, disease states include, but are not limited to diabetes, glycogen storage diseases, obesity, polycystic ovarian syndrome, hypertension, atherosclerosis and other diseases of insulin-resistance).

The rationale for the therapeutic use of SYNIP to design or discover treatment for these diseases is based upon the general disregulation of glucose transport in such states. Numerous studies have shown that the stimulation of glucose transport by insulin is significantly reduced in Type II diabetes and other states of insulin resistance. Thus, pharmacological or genetic approaches to

alleviating this deficiency will have a major impact on the diseases described above.

One aspect of the invention is to provide purified SYNIPs. The purified proteins may be obtained from either recombinant cells or naturally occurring cells. The purified SYNIPs of the invention may be mammalian in origin. Primate, including human-derived SYNIPs are examples of the various SYNIPs specifically provided for. The invention also provides allelic variants and biologically active derivatives of naturally occurring SYNIPs.

Another aspect of the invention is to provide polynucleotides encoding the SYNIPs of the invention and to provide polynucleotides complementary to polynucleotide coding strand. The polynucleotides of the invention may be used to provide for the recombinant expression of SYNIPs. The polynucleotides of the invention may also be used for genetic therapy purposes so as to treat diseases related to intracellular receptors that bind ligands that bind to SYNIPs, used in the detection of genetic deletions of the polynucleotide, subcellular localization of the polypeptide, and isolation of discrete classes of RNA. The invention also provides polynucleotides for use as hybridization probes and amplification primers for the detection of naturally occurring polynucleotides encoding SYNIPs.

Another aspect of the invention is to provide antibodies capable of binding to the SYNIPs of the invention. The antibodies may be polyclonal or monoclonal. The invention also provides methods of using the subject antibodies to detect and measure expression of SYNIPs either *in vitro* or *in vivo*, or for detecting proteins that interact with SYNIPs, or molecules that regulate any of the activities of SYNIPs.

Another aspect of the invention is to provide assays for the detection or screening of therapeutic compounds that interfere with the interaction between SYNIPs and syntaxin-4 (or other ligands that bind to SYNIPs). The assays of the invention comprise the step of measuring the effect of a compound of interest on binding between SYNIPs and syntaxin-4 (or other ligands that bind to SYNIPs). Binding may be measured in a variety of ways, including the use of labeled SYNIPs or labeled ligands.

Another aspect of the invention is to provide assays for the discovery of proteins that interact directly or indirectly with SYNIPs. The assays of the

invention comprise a method for detecting such interactions in cells, or in biochemical assays. These interactions may be detected in a variety of ways, including the use of the cDNA encoding SYNIPs, or SYNIPs themselves, or fragments or modifications thereof.

The foregoing is not intended and should not be construed as limiting the invention in any way. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All U.S. patents and all publications mentioned herein are incorporated in their entirety by reference thereto.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Cloning, characterization of SYNIP expression and specificity of binding. A) Deduced amino acid sequences of the single open reading frame in the isolated SYNIP cDNA. B) Predicted structural organization of SYNIP functional domains. The numbers on the top indicate the amino acid residues that define the boundaries of these domains. C) Northern blot analysis of SYNIP mRNA expression in mouse tissues. The mouse multiple tissue mRNA blot was probed with the coding sequences of SYNIP cDNA. H, heart; Br, brain; Sp, spleen; Lu, lung; Li, liver; Sk, skeletal muscle; K, kidney; Te, testis. D) Specificity of SYNIP/WT and SYNIP/CT binding to Syntaxin 4. Cell lysates from 293T cells overexpressing Flag-tagged wild type SYNIP (SYNIP/WT) or the carboxyl terminal SYNIP (SYNIP/CT) were incubated with equal amounts of GST (lane 1), GST-Syn1A (lane 2), GST-Syn1B (lane 3), GST-Syn2 (lane 4), GST-Syn3 (lane 5) and GST-Syn4 (lane 6) proteins immobilized on Glutathion-agarose beads. The retained proteins were immunoblotted with anti-Flag antibody. The SYNIP and cDNA sequence have been deposited in GeneBank. Accession number XXXXXX.

Figure 2. Insulin stimulation induces a dissociation of SYNIP from syntaxin-4 *in vivo*. CHO/IR cells were transfected with the full-length SYNIP (SYNIP/WT), the amino terminal SYNIP domain (SYNIP/NT) or the carboxyl terminal SYNIP domain (SYNIP/CT) and stimulated with and without insulin.

A) Cell lysates were prepared and immunobloted with the Flag antibody. B) Cell lysates were immunoprecipitated with a syntaxin-4 antibody and immunoblotted with the Flag antibody. C) The immunoprecipitates in (B) were immunoblotted with the syntaxin-4 antibody.

Figure 3. Insulin stimulation results in a decreased affinity of SYNIP for syntaxin-4. CHO/IR cells were transfected with the full-length SYNIP (SYNIP/WT), the amino terminal SYNIP domain (SYNIP/NT) or the carboxyl terminal SYNIP domain (SYNIP/CT) and stimulated with and without insulin. A) Cell lysates were prepared and immunobloted with the Flag antibody. B) Cell lysates were incubated with the GST-Syn4 fusion protein and the resultant precipitates were immunoblotted with the Flag antibody. C) Cell lysates were incubated with the GST-SYNIP fusion protein and the resultant precipitates were immunoblotted with the syntaxin-4 antibody. D) Cell lysates were incubated with various amounts of the GST-Syn4 fusion protein and the resultant precipitates were immunoblotted with the Flag antibody.

Figure 4. Insulin induces dissociation of the SYNIP:syntaxin-4 complex in differentiated 3T3L1 adipocytes. Differentiated 3T3L1 adipocytes were transfected with the full-length SYNIP (SYNIP/WT) or the carboxyl terminal SYNIP domain (SYNIP/CT) and stimulated with and without insulin. A) Cell lysates were prepared and immunobloted with the Flag antibody. B) The cell lysates were then incubated with the GST-Syn4 fusion protein and the resultant precipitates were immunoblotted with the Flag antibody.

Figure 5. Expression of a dominant-interfering mutant of SYNIP inhibits insulin-stimulated glucose transport. A) Differentiated 3T3L1 adipocytes were electroporated with various amounts of pcDNA3.1-LacZ and transfection/expression efficiency assessed by X-Gal staining for β-galactosidase expression. Under each electroporation condition the total amount of plasmid DNA was 600 μg maintained by the addition of the pcDNA3.1 empty vector. B) Differentiated 3T3L1 adipocytes were electroporated with either empty vector or various SYNIP cDNA constructs and the rate of basal (open bars) and insulin-stimulated (solid bars) 2-deoxyglucose transport was determined.

Figure 6. Expression of a dominant-interfering mutant of SYNIP inhibits insulin-stimulated GLUT4 but not GLUT1 translocation. A) Differentiated 3T3L1 adipocytes were co-transfected with GLUT4-eGFP and various SYNIP cDNAs. B) Differentiated 3T3L1 adipocytes were co-transfected with eGFP-GLUT1 and various SYNIP cDNAs. The subcellular localization of GLUT4-eGFP and eGFP-GLUT1 was determined in control and insulin-stimulated cells by confocal fluorescence microscopy.

Figure 7. Hypothetical model for the insulin-dependent regulation of SYNIP function and GLUT4 translocation.

DETAILED DESCRIPTION OF THE INVENTION

Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutshcer, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis et al., 1990, Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique*, 2nd ed. (R.I. Freshney, 1987, Liss, Inc. New York, NY), and *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, NJ).

In one aspect, the present invention provides novel isolated and purified polynucleotides, hereinafter referred to as syntaxin-4 interacting ("SYNIP") protein genes, encoding SYNIPs. The term "syntaxin-4" is used broadly herein. Unless noted otherwise, the term "syntaxin-4" include, but is not limited to, any natural mammalian-derived form of syntaxin-4 and the like. It is preferred that the term syntaxin-4 include primates and humans. Also, the term "interacting" is used broadly herein. Unless noted otherwise, the term "interacting" includes, but is not limited to, binding, affecting, and regulating.

The polynucleotides provided for may encode complete SYNIPs or portions thereof. The polynucleotides of the invention may be produced by a variety of methods including *in vitro* chemical synthesis using well-known solid phase synthesis technique, by cloning or combinations thereof. The polynucleotide of the invention may be derived from cDNA or genomic libraries. Persons of ordinary skill in the art are familiar with the degeneracy of the genetic code and may readily design polynucleotides that encode SYNIPs that have either partial or polynucleotide sequence homology to naturally occurring polynucleotide sequences encoding SYNIPs. The polynucleotides of the invention may be single stranded or double stranded. Polynucleotide complementary to polynucleotides encoding SYNIPs are also provided.

Polynulceotide encoding a SYNIP can be obtained from cDNA libraries prepared from tissue believed to possess SYNIP mRNA and to express it at a detectable level. For example, cDNA library can be constructed by obtaining polyadenylated mRNA from a cell line known to express SYNIP, and using the mRNA as a template to synthesize double stranded cDNA.

Libraries, either cDNA or genomic, are screened with probes designed to identify the gene of interest or the protein encoded by it. For cDNA expression libraries, suitable probes include monoclonal and polyclonal antibodies that recognize and specifically bind to a SYNIP. For cDNA libraries, suitable probes include carefully selected oligonucleotide probes (usually of about 20-80 bases in length) that encode known or suspected portions of a SYNIP from the same or different species, and/or complementary or homologous cDNAs or fragments thereof that encode the same or a similar gene, and/or homologous genomic DNAs or fragments thereof. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures as described in Chapters 10-12 of Sambrook et al., *Molecular Cloning: A Laboratory Manual*, New York, Cold Spring Harbor Laboratory Press, 1989).

A preferred method of practicing this invention is to use carefully selected oligonucleotide sequences to screen cDNA libraries from various tissues. The oligonucleotide sequences selected as probes should be sufficient in length and sufficiently unambiguous that false positives are minimized. The actual nucleotide sequence(s) is/are usually designed based on regions of a SYNIP that have the

least codon redundance. The oligonucleotides may be degenerate at one or more positions. The use of degenerate oligonucleotides is of particular importance where a library is screened from a species in which preferential codon usage is not known.

The oligonucleotide must be labeled such that it can be detected upon hybridization to DNA in the library being screened. The preferred method of labeling is to use ATP (eg, T32P) and polynucleotide kinase to radiolabel the 5′ end of the oligonucleotide. However, other methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labeling.

cDNAs encoding SYNIPs can also be identified and isolated by other known techniques of recombinant DNA technology, such as by direct expression cloning or by using the polymerase chain reaction (PCR) as described in US Patent No. 4,683,195, in Section 14 of Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory Press, New York, 1989, or in Chapter 15 of *Current Protocols in Molecular Biology*, Ausubel et al., eds., Green Publishing Associates and Wiley-Interscience, 1991. This method requires the use of oligonucleotide probes that will hybridize to DNA encoding a SYNIP.

In a preferred embodiment, the invention comprises DNA sequences substantially similar to those shown in SEQ ID 1 or 6 (mouse SYNIP polynucleotides) and SEQ ID 3 or 4 (human SYNIP polyneucleotides). As defined herein, "substantially similar" includes identical sequences, as well as deletions, substitutions or additions to a DNA, RNA or protein sequence that maintain the function of the protein product and possess similar zinc-binding motifs. Preferably, the DNA sequences according to the invention consist essentially of the DNA sequence of SEQ ID 1, 3, 4, or 6. These novel purified and isolated DNA sequences can be used to direct expression of SYNIP and for mutational analysis of SYNIP function.

Mutated sequences according to the invention can be identified in a routine manner by those skilled in the art using the teachings provided herein, and techniques well-known in the art.

In a preferred embodiment, the present invention comprises a nucleotide sequence that hybridizes to the nucleotide sequence shown in SEQ ID 1, 3, 4, or 6 under high stringency hybridization conditions. As used herein, the term "high stringency hybridization conditions" refers to hybridization at 65°C in a low salt hybridization buffer to the probe of interest at 2×10^8 cpm/μg for between about 8 hours to 24 hours, followed by washing in 1% SDS, 20 mM phosphate buffer and 1 mM EDTA at 65°C, for between about 30 minutes to 4 hours. In a preferred embodiment, the low salt hybridization buffer comprises between, 0.5-10% SDS, and 0.05 M and 0.5 M sodium phosphate. In a most preferred embodiment, the low salt hybridization buffer comprises, 7% SDS, and 0.125 M sodium phosphate.

The polynucleotides of the invention have a variety of uses, some of which have been indicated or will be addressed in greater detail, *infra*. The particular uses for a given polynucleotide depend, in part, on the specific polynucleotide embodiment of interest. The polynucleotides of the invention may be used as hybridization probes to recover SYNIP encoding polynucleotides or a portion thereof from genetic libraries. The polynucleotides of the invention may also be used as primers for the amplification of SYNIP encoding polynucleotides or a portion thereof through the polymerase chain reaction (PCR) and other similar amplification procedures. The polynucleotides of the invention may also be used as probes and amplification primers to detect mutations in SYNIP encoding polynucleotides or a portion thereof that have been correlated with diseases, particularly diseases related to overexpression or underexpression of ligands for SYNIP.

The invention also provides a variety of polynucleotide expression vectors, comprising SYNIP encoding polynucleotides or a portion thereof or a sequence substantially similar to it subcloned into an extra-chromosomal vector. This aspect of the invention allows for *in vitro* expression of SYNIP encoding polynucleotides, thus permitting an analysis of SYNIP encoding polynucleotides regulation and SYNIP structure and function. As used herein, the term "extra-chromosomal vector" includes, but is not limited to, plasmids, bacteriophages, cosmids, retroviruses and artificial chromosomes. In a preferred embodiment, the extra-chromosomal vector comprises an expression vector that allows for SYNIP

production when the recombinant DNA molecule is inserted into a host cell. Such vectors are well-known in the art and include, but are not limited to, those with the T3 or T7 polymerase promoters, the SV40 promoter, the CMV promoter, or any promoter that either can direct gene expression, or that one wishes to test for the ability to direct gene expression.

In a preferred embodiment, the subject expression vectors comprise a polynucleotide sequence encoding a SYNIP in functional combination with one or more promoter sequences so as to provide for the expression of the SYNIP (or an anti-sense copy of the sequence suitable for inhibition of expression of an endogenous gene). The vectors may comprise additional polynucleotide sequences for gene expression, regulation, or the convenient manipulation of the vector, such additional sequences include terminators, enhancers, selective markers, packaging sites, and the like. Detailed description of polynucleotide expression vectors and their use can be found in, among other places *Gene Expression Technology: Methods in Enzymology*, Volume 185, Goeddel, ed., Academic Press Inc., San Diego, CA (1991), *Protein Expression in Animal Cells*, Roth, ed., Academic Press, San Diego, CA (1994).

The polynucleotide expression vectors of the invention have a variety of uses. Such uses include the genetic engineering of host cells to express SYNIPs. In a further aspect, the present invention provides recombinant host cells that are stably transfected with a recombinant DNA molecule comprising SYNIP encoding polynucleotides subcloned into an extra-chromosomal vector. The host cells of the present invention may be of any type, including, but not limited to, bacterial, yeast, and mammalian cells. Transfection of host cells with recombinant DNA molecules is well-known in the art (Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Press, 1989) and, as used herein, includes, but is not limited to calcium phosphate transfection, dextran sulfate transfection, electroporation, lipofection and viral infection. This aspect of the invention allows for *in vitro* and *in vivo* expression of SYNIP and its gene product, or a portion of SYNIP and its gene product, thus enabling high-level expression of SYNIP or a portion thereof.

Other uses of the polynucleotide expression vectors, discussed in greater detail, *infra*, include, their use for genetic therapy for diseases and conditions in

which it may be desirable use to express SYNIPs at levels greater than naturally occurring expression levels. Alternatively, it may be desirable to use the subject vectors for anti-sense expression to reduce the naturally occurring levels of SYNIP.

In another aspect, the present invention provides a substantially purified recombinant protein comprising a polypeptide substantially similar to the SYNIP shown in SEQ ID 2 or 5. Furthermore, this aspect of the invention enables the use of SYNIP in several *in vitro* assays described below. As used herein, the term "substantially similar" includes deletions, substitutions and additions to the sequences of SEQ ID 2 or 5 introduced by any *in vitro* means. As used herein, the term "substantially purified" means that the protein should be free from detectable contaminating protein, but the SYNIP may be co-purified with an interacting protein, or as an oligomer. In a most preferred embodiment, the protein sequence according to the invention comprises an amino acid sequence of SEQ ID 2 or 5. Mutated sequences according to the invention can be identified in a routine manner by those skilled in the art using the teachings provided herein and techniques well-known in the art. This aspect of the invention provides a novel purified protein that can be used for *in vitro* assays, and as a component of a pharmaceutical composition for GLUT4 translocation modification, described *infra*.

SYNIPs may be used to discover molecules that interfere with its activities. For example, molecules that prevent the binding of SYNIP to Syntaxin-4 in insulin responsive tissues, thus increasing glucose transport. Additionally, SYNIPs may be used to find other proteins that can directly interact with it, representing additional important regulators of glucose transport.

The SYNIPs of the present invention have the biological activity of binding to syntaxin-4. The SYNIP of the invention may be isolated from a variety of mammalian animal species. Preferred mammalian species for isolation are primates and humans. The invention also contemplates allelic variants of SYNIP. SYNIPs may be prepared from a variety of mammalian tissues, however cell lines established from insulin responsive tissues are preferred non-recombinant sources of SYNIPs. Preferably SYNIPs are obtained from recombinant host cells genetically engineered to express significant quantities of SYNIPs. SYNIPs may

be isolated from non-recombinant or recombinant cells in a variety of ways well-known to a person of ordinary skill in the art.

The term "SYNIP" as used herein refers not only to proteins having the amino acid residue sequence of naturally occurring SYNIPs, but also refers to functional derivatives and variants of naturally occurring SYNIP. A "functional derivative" of a native polypeptide is a compound having a qualitative biological activity in common with the native SYNIP. Thus, a functional derivative of a native SYNIP is a compound that has a qualitative biological activity in common with a native SYNIP, eg, binding to syntaxin-4 and other cognate ligands. "Functional derivatives" include, but are not limited to, fragments of native polypeptides from any animal species (including human), and derivatives of native (human and non-human) polypeptides and their fragments, provided that they have a biological activity in common with a respective native polypeptide. "Fragments" comprise regions within the sequence of a mature native polypeptide. The term "derivative" is used to define amino acid sequence and glycosylation variants, and covalent modifications of a native polypeptide, whereas the term "variant" refers to amino acid sequence and glycosylation variants within this definition. Preferably, the functional derivatives are polypeptides which have at least about 65% amino acid sequence identity, more preferably about 75% amino acid sequence identity, even more preferably at least 85% amino acid sequence identity, most preferably at least about 95% amino acid sequence identity with the sequence of a corresponding native polypeptide. Most preferably, the functional derivatives of a native SYNIP retain or mimic the region or regions within the native polypeptide sequence that directly participate in ligand binding. The phrase "functional derivative" specifically includes peptides and small organic molecules having a qualitative biological activity in common with a native SYNIP.

"Identity" or "homology" with respect to a native polypeptide and its functional derivative is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues of a corresponding native polypeptide, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. Neither N- or

C-terminal extensions nor insertions shall be construed as reducing identity or homology. Methods and computer programs for the alignment are well-known in the art.

Amino acid sequence variants of native SYNIPs and SYNIP fragments are prepared by methods known in the art by introducing appropriate nucleotide changes into a native or variant SYNIP encoding DNA, or by *in vitro* synthesis of the desired polypeptide. There are two principal variables in the construction of amino acid sequence variants: the location of the mutation site and the nature of the mutation. With the exception of naturally-occurring alleles, which do not require the manipulation of the DNA sequence encoding the SYNIP, the amino acid sequence variants of SYNIP are preferably constructed by mutating the DNA, either to arrive at an allele or an amino acid sequence variant that does not occur in nature.

Alternatively or in addition, amino acid alterations can be made at sites that differ in SYNIPs from various species, or in highly conserved regions, depending on the goal to be achieved.

Sites at such locations will typically be modified in series, eg, by (1) substituting first with conservative choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue or residues, or (3) inserting residues of the same or different class adjacent to the located site, or combinations of options 1-3.

One helpful technique is called "alanine scanning," Cunningham and Wells, *Science*, 1989;244:1081-1085. Here, a residue or group of target resides is identified and substituted by alanine or polyalanine. Those domains demonstrating functional sensitivity to the alanine substitutions are then refined by introducing further or other substituents at or for the sites of alanine substitution.

After identifying the desired mutation(s), the gene encoding a SYNIP variant can, for example, be obtained by chemical synthesis.

More preferably, DNA encoding a SYNIP amino acid sequence variant is prepared by site-directed mutagenesis of DNA that encodes an earlier prepared variant or a nonvariant version of SYNIP. Site-directed (site-specific) mutagenesis allows the production of SYNIP variants through the use of specific oligonucleotide sequences that encode the DNA sequence of the desired mutation,

as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 20 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered. In general, the techniques of site-specific mutagenesis are well-known in the art, as exemplified by publications such as, Edelman et al., *DNA*, 1983;2:183. As will be appreciated, the site-specific mutagenesis technique typically employs a phage vector that exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. This and other phage vectors are commercially available, and their use is well-known to those skilled in the art. A versatile and efficient procedure for the construction of oligodeoxyribonucleotide directed site-specific mutations in DNA fragments using M13-derived vectors was published by Zoller M.J. and Smith M., *Nucleic Acids Res.*, 1982;10:6487-6500). Also, plasmid vectors that contain a single-stranded phage origin of replication, Veira et al., *Meth. Enzymol.*, 1987;153:3, may be employed to obtain single-stranded DNA. Alternatively, nucleotide substitutions are introduced by synthesizing the appropriate DNA fragment *in vitro*, and amplifying it by PCR procedures known in the art.

In general, site-specific mutagenesis may be performed by first obtaining a single-stranded vector that includes within its sequence a DNA sequence that encodes the relevant protein. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example, by the method of Crea et al., *Proc. Natl. Acad. Sci., USA*, 1978;75:5765. This primer is then annealed with the single-stranded protein sequence-containing vector, and subjected to DNA-polymerizing enzymes such as, *E. coli* polymerase I Klenow fragment, to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desires mutation. This heteroduplex vector is then used to transform appropriate host cells such as HB101 cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement. Thereafter, the mutated region may be removed and placed in an appropriate expression vector for protein production.

The PCR technique may also be used in creating amino acid sequence variants of a SYNIP. When small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, one of the primers is designed to overlap the position of the mutation and to contain the mutation; the sequence of the other primer must be identical to a stretch of sequence of the opposite strand of the plasmid, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 200 nucleotides from that of the first, such that in the end the entire amplified region of DNA bounded by the primes can be easily sequenced. PCR amplification using a primer pair like the one just described results in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone.

Further details of the foregoing and similar mutagenesis techniques are found in general textbooks, such as, for example, Sambrook et al., *Molecular Cloning: H Laboratorv Manual*, 2nd edition, Cold Spring Harbor Press, Cold Spring Harbor (1989), and *Current Protocols in Molecular Biology*, Ausubel et al., eds., John Wiley and Sons (1995).

Naturally-occurring amino acids are divided into groups based on common side chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophobic: cys, ser. tier;

(3) acidic: asp, glu;

(4) basic: asn, gin, his, lys, erg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, pine.

Conservative substitutions involve exchanging a member within one group for another member within the same group, whereas non-conservative substitutions will entail exchanging a member of one of these classes for another.

Variants obtained by non-conservative substitutions are expected to result in significant changes in the biological properties/function of the obtained variant, and may result in SYNIP variants which block SYNIP biological activities, ie, ligand binding. Amino acid positions that are conserved among various species are generally substituted in a relatively conservative manner if the goal is to retain biological function.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably about 1 to 10 residues, and typically are contiguous. Deletions may be introduced into regions not directly involved in ligand binding.

Amino acid insertions include amino- and/or carboxyl terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (ie, insertions within the SYNIP amino acid sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5 residues, more preferably 1 to 3 residues. Examples of terminal insertions include the SYNIPs with an N-terminal methionyl residue, an artifact of direct expression in bacterial recombinant cell culture, and fusion of a heterologous N-terminal signal sequence to the N-terminus of the SYNIP to facilitate the secretion of the mature SYNIP from recombinant host cells. Such signal sequences will generally be obtained from, and thus homologous to, the intended host cell species. Suitable sequences include STII or Ipp for *E. coli*, alpha factor for yeast, and viral signals such as herpes gD for mammalian cells. Other insertional variants of the native SYNIP molecules include the fusion of the N- or C-terminus of an SYNIP to immunogenic polypeptides, eg, bacterial polypeptides such as betalactamase or an enzyme encoded by the *E. coli* trp locus, or yeast protein, and C-terminal fusions with proteins having a long half-life such as immunoglobulin regions (preferably immunoglobulin constant regions), albumin, or ferritin, as described in PCT published application WO 89/02922.

Since it is often difficult to predict in advance the characteristics of a variant SYNIP, it will be appreciated that screening will be needed to select the optimum variant. For this purpose biochemical screening assays, such as those described herein below, will be readily available.

In a further aspect, the present invention provides antibodies and methods for detecting antibodies that selectively bind polypeptides with an amino acid sequence substantially similar to the amino acid sequence of SEQ ID 2 or 5. As discussed in greater detail, *infra*, the antibody of the present invention can be a polyclonal or a monoclonal antibody, prepared by using all or part of the sequence of SEQ ID 2 or 5, or modified portions thereof, to elicit an immune response in a host animal according to standard techniques (Harlow and Lane (1988), eds., *Antibody: A Laboratory Manual*, Cold Spring Harbor Press). In a preferred embodiment, the entire polypeptide sequence of SEQ ID 2 is used to elicit the production of polyclonal antibodies in a host animal.

The method of detecting SYNIP antibodies comprises contacting cells with an antibody that recognizes SYNIP and incubating the cells in a manner that allows for detection of the SYNIPantibody complex. Standard conditions for antibody detection of antigen can be used to accomplish this aspect of the invention (Harlow and Lane, 1988). This aspect of the invention permits the detection of SYNIP protein both *in vitro* and *in vivo*.

The subject invention provides methods for the treatment of a variety of diseases characterized by undesirably abnormal levels of glucose or abnormal GLUT4 translocation. Diseases may be treated through either *in vivo* or *in vitro* genetic therapy. Protocols for genetic therapy through the use of viral vectors can be found, among other places, in *Viral Vector Gene Therapy and Neuroscience Applications*, Kaplit and Lowry, Academic Press, San Diego (1995). The genetic therapy methods of the invention comprise the step of introducing a vector for the expression of SYNIP (or inhibitory anti-sense RNA) into a patient cell. The patient cell may be either in the patient, ie, *in vivo* genetic therapy, or external to the patient and subsequently reintroduced into the patient, ie, *in vitro* genetic therapy. Diseases that may be treated by the subject genetic therapy methods include, but are not limited to diabetes, glycogen storage diseases, obesity, polycystic ovarian syndrome, hypertension, atherosclerosis and other diseases of insulin-resistance.

In a preferred aspect of the invention, a method is provided for protecting mammalian cells from abnormal levels of glucose or abnormal GLUT4 translocation, comprising introducing into mammalian cells an expression vector

comprising a DNA sequence substantially similar to the DNA sequence shown in SEQ ID 1 or 4, that is operatively linked to a DNA sequence that promotes the expression of the DNA sequence and incubating the cells under conditions wherein the DNA sequence of SEQ ID 1 or 4 will be expressed at high levels in the mammalian cells. Suitable expression vectors are as described above. In a preferred embodiment, the coding region of the human SYNIP gene (SEQ ID 4) is subcloned into an expression vector under the transcriptional control of the cytomegalovirus (CMV) promoter to allow for constitutive SYNIP gene expression.

In another preferred aspect of the present invention, a method is provided for treating or preventing abnormal levels of glucose or abnormal GLUT4 translocation, comprising introducing into mammalian tumor cells an expression vector comprising a DNA that is antisense to a sequence substantially similar to the DNA sequence shown in SEQ ID 1 or 4 that is operatively linked to a DNA sequence that promotes the expression of the antisense DNA sequence. The cells are then grown under conditions wherein the antisense DNA sequence of SEQ ID 1 or 4 will be expressed at high levels in the mammalian cells.

In a most preferred embodiment, the DNA sequence consists essentially of SEQ ID 1 or 4. In a further preferred embodiment, the expression vector comprises an adenoviral vector wherein SYNIP cDNA is operatively linked in an antisense orientation to a cytomegalovirus (CMV) promoter to allow for constitutive expression of the SYNIP antisense cDNA in a host cell. In a preferred embodiment, the SYNIP adenoviral expression vector is introduced into mammalian insulin-sensitive cells by injection into a mammal.

Another aspect of the invention is to provide assays useful for determining if a compound of interest can bind to SYNIPs so as to interfere with the binding of syntaxin-4 (or other ligands) to the v- and t-SNAREs. The assay comprises the steps of measuring the binding of a compound of interest to a SYNIP . Either the SYNIP or the compound of interest to be assayed may be labeled with a detectable label, eg, a radioactive or fluorescent label, so as to provide for the detection of complex formation between the compound of interest and the SYNIP. In another embodiment of the subject assays, the assays involve measuring the interference, ie, competitive binding, of a compound of interest with the binding interaction

between a SYNIP and syntaxin-4 (or another ligand already known to bind to SYNIP). For example, the effect of increasing quantities of a compound of interest on the formation of complexes between radioactivity labeled syntaxin-4 and an SYNIP may be measured by quantifying the formation of labeled ligand-SYNIP complex formation.

Polyclonal antibodies to SYNIPs generally are raised in animals by multiple subcutaneous (se) or intraperitoneal (ip) injections of a SYNIP and an adjuvant. It may be useful to conjugate the SYNIP or a fragment containing the target amino acid sequence to a protein that is immunogenic in the species to be immunized, eg, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine resides), glutaraldehyde, succinic anhydride, $SOCl_2$, or R_1~N=C=NR, where R and R_1 are different alkyl groups.

Animals are immunized against the immunogenic conjugates or derivatives by combing 1 mg or 1 fig of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for anti-SYNIPs antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal boosted with the conjugate of the same SYNIP, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such a alum are used to enhance the immune response.

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, ie, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies. For example, the

anti-SYNIP monoclonal antibodies of the invention may be made using the hybridoma method first described by Kohler & Milstein, *Nature*, 1975;256:495, or may be made by recombinant DNA methods [Cabilly et al, US Pat. No. 4,816,567].

In the hybridoma method, a mouse or other appropriate host animal, such a hamster is immunized as herein above described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Coding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)].

The anti-SYNIP specific antibodies of the invention have a number of uses. The antibodies may be used to purify SYNIPs from either recombinant or non-recombinant cells. The subject antibodies may be used to detect and/or quantify the presence of SYNIPs in tissue samples, eg, from blood, skin, and the like. Quantitation of SYNIPs may be used diagnostically for those diseases and physiological or genetic conditions that have been correlated with particular levels of SYNIP expression levels.

In a further aspect, the present invention provides a diagnostic assay for detecting cells containing SYNIP polynucleotide deletions, comprising isolating total genomic DNA from the cell and subjecting the genomic DNA to PCR amplification using primers derived from the DNA sequence of SEQ ID 1 or 4.

This aspect of the invention enables the detection of SYNIP polynucleotide deletions in any type of cell, and can be used in genetic testing or as a laboratory tool. The PCR primers can be chosen in any manner that allows the amplification of a SYNIP polynucleotide fragment large enough to be detected by gel electrophoresis. Detection can be by any method, including, but not limited to ethidium bromide staining of agarose or polyacrylamide gels, autoradiographic detection of radio-labeled SYNIP gene fragments, Southern blot hybridization, and DNA sequence analysis. In a preferred embodiment, detection is accomplished by polyacrylamide gel electrophoresis, followed by DNA sequence analysis to verify the identity of the deletions. PCR conditions are routinely

determined based on the length and base-content of the primers selected according to techniques well-known in the art (Sambrook et al., 1989).

An additional aspect of the present invention provides a diagnostic assay for detecting cells containing SYNIP polynucleotide deletions, comprising isolating total cell RNA and subjecting the RNA to reverse transcription-PCR amplification using primers derived from the DNA sequence of SEQ ID 1 or 4. This aspect of the invention enables the detection of SYNIP deletions in any type of cell, and can be used in genetic testing or as a laboratory tool.

Reverse transcription is routinely accomplished via standards techniques (Ausubel et al., in *Current Protocols in Molecular Biology*, ed. John Wiley and Sons, Inc., 1994) and PCR is accomplished as described above.

In another aspect, the present invention provides methods of isolating RNA containing stretches of polyA (adenine), polyC (cytosine) or polyU (uridine) residues, comprising contacting an RNA sample with SYNIP, incubating the RNA-SYNIP mixture with an antibody that recognizes the SYNIP polypeptide, isolating the antibody-SYNIP-RNA complexes, and purifying the RNA away from the antibody-SYNIP complex. This aspect of the invention provides a novel in vitro method for isolating a discrete class of RNA. In a preferred embodiment, the RNA sample is contacted with SYNIP in the presence (for preferential isolation of polyA and polyC-containing RNAs), or absence (for preferential isolation of polyU-containing RNAs), of a reducing agent. Preferred reducing agents for use in this aspect of the invention include, but are not limited to DTT and β-mercaptoethanol. The reducing agents are preferably used at a concentration of between about 50 nM and 1 M. Isolation of antibody-SYNIP-RNA complexes can be accomplished via standard techniques in the art, including, but not limited to the use of Protein-A conjugated to agarose or cellulose beads.

The present invention may be better understood with reference to the accompanying examples that are intended for purposes of illustration only and should not be construed to limit the scope of the invention, as defined by the claims appended hereto.

EXAMPLES

Example 1

Summary

Insulin-stimulated glucose transport and GLUT4 translocation require specific interactions between the v-SNARE, VAMP2, and the t-SNARE, syntaxin- 4. However, insulin does not directly effect these or any other SNARE-like molecules identified to date. As shown in the following example, a novel syntaxin-4 binding protein, SYNIP, was isolated which specifically interacted with syntaxin-4 and was only expressed in cells that displayed insulin-responsive glucose transport and GLUT4 translocation. Insulin induced a dissociation of the SYNIP:syntaxin-4 complex due to a decreased binding affinity of SYNIP for syntaxin-4. In contrast, the binding of the carboxyl terminal SYNIP domain was refractive to insulin stimulation but inhibited glucose transport and GLUT4 translocation. These data identify SYNIP as the first insulin-regulated SNARE-like protein directly involved in the regulation of glucose transport and GLUT4 vesicle translocation.

Experimental Procedures

Materials

The Flag M_2 monoclonal antibody was obtained from Kodak and the syntaxin-4 sheep polyclonal antibody was isolated as previously described (Olson A.L., Knight J.B., and Pessin J.E., "Syntaxin 4, VAMP2, and/or VAMP3/cellubrevin are functional target membrane and vesicle SNAP receptors for insulin-stimulated GLUT4 translocation in adipocytes." *Mol Cell Biol*, 1997;17:2425-2435). The β-galactosidase expression plasmid (pcDNA3.1/his/LacZ) was purchased from Invitrogen. SuperSigal ULTRA Enhanced Chemiluminescent (ECL) reagents, and the secondary anti-sheep and anti-rabbit IgG-HRP were from Pierce. ECL western blotting reagents and [α-^{32}P]dCTP were purchased from Amersham Life Science. All restriction enzymes, cell culture media, and reagents were from GIBCO BRL. All other reagents were obtained from Sigma Chemical Co. unless specifically noted.

Isolation of SYNIP cDNA by yeast two-hybrid screening

The coding region of the cytoplasmic domain of syntaxin-4 (residue 2-274) was amplified by PCR from the plasmid carrying syntaxin-4 cDNA using the primers 5'CGGGATCCTGCGCGACAGGACCCATG 3' and 5' GGTCGACCTTTTTCTTCCTCGC 3'. The PCR product was then subcloned into BamHI-SalI site of the bait vector pGBT9 (Clontech), in frame with GAL4 DNA-binding domain. To screen for syntaxin-4 binding proteins, yeast strain Y190 was sequentially transformed with the bait DNA and then the yeast two-hybrid cDNA library constructed from 3T3L1 adipocyte mRNA as previously described (Printen J.A., Brady M.J., and Saltiel A.R., "PTG, a protein phosphatase 1-binding protein with a role in glycogen metabolism." *Science*, 1997;275:1475-1478). The transformation was plated onto synthetic media lacking tryptophan, leucine and histidine and containing 25 mM 3-aminotriazole (Sigma) and incubated at 30°C. Colonies which appeared after 5-7 days of incubation were analyzed for β-galactosidase activity by plating onto the media containing X-Gal. The prey cDNAs were recovered from the strongest hits and were subjected to DNA sequencing. All sequences were analyzed by BLAST search, Protein tool and COILS 2.2 programs.

Northern blot analysis

The 1.67 kb of the SYNIP cDNA coding sequences were radiolabeled with [α-^{32}P]dCTP using a random hexamer labelling kit, and the probe was purified with a QIAquick Nucleotide Removal Kit (QIAGEN). The probe was hybridized with a Northern blot containing 2 mg of purified poly A$^+$ RNA isolated from various mouse tissues in ExpressHyb hybridization solution (Clontech) for 16 hours at 65°C. The blot was then extensively washed followed manufacturer's recommendation and subjected to autoradiography.

Expression Constructs

The coding region of SYNIP cDNA was PCR amplified from a plasmid containing 2.6 kb SYNIP cDNA with a pair of oligos: 5'GTACTGACCCGGGAATTCGAAAGCATGAGTGATGGTACAGC3' and

5'GTCGACGCGGCCGCTCGAGCTACTTGTCATCGTCGTCCTTGTA GTCGCTTTTCGGGTCTGTTAGCTCTCTG3'. The 3' end of the primer incorporated sequences encoding for a eight-amino acid flag epitope. The PCR product was cloned into pCR2.1 vector (Invitrogen). To construct the SYNIP/WT mammalian expression plasmid, the full length carboxyl terminal Flag-tagged SYNIP was subcloned into EcoR1/Xho1 sites of the pcDNA3 vector (Invitrogen). To construct SYNIP/NT (residue 1-301) deletion mutant, the insert was first generated by PCR with primers 5'ACTGAATTCATGAGTGATGGTA CTGCTTCTGC3' and 5'ATCCTCGAGCACTTCATCTGCTTCTAGAG 3' and cloned into EcoRI/XhoI sites of a pcDNA3 vector containing a Flag tag immediately downstream of XhoI site. The SYNIP/NT construct was obtained by switching an internal EcoRI/XbaI fragment with the same fragment from the SYNIP/WT plasmid so that it contained the wild type Kozak sequences. To construct the SYNIP/CT mutant, the original two hybrid cDNA was subcloned into EcoRI/SalI sites of pFlag-CMV2 vector (Kodak).

The GLUT4-eGFP fusion construct was prepared by subcloning the rat GLUT4 cDNA into the pEGFP vector (Clontech) at the 5' BamHI and 3' HindIII sites. The GLUT4 cDNA was put in frame with the EGFP cDNA by excising the 200 bp BglII-AgeI fragment and replacing it with the BglII/AgeI digested PCR fragment generated by amplification of the rat GLUT4 cDNA using primers 5'CTTCATCTTCACCTTCCTAA3' and 5'GGTGGCGACCGGTA CGTCATTCTCATCTGG3'. The fusion protein is the contiguous sequence of GLUT4 with 5 additional amino acids (Val-Pro-Val-Ala-Thr) connecting it to EGFP. The resultant GLUT4-eGFP was subcloned into pcDNA3 vector using the 5' HindIII and 3' XbaI sites. The eGFP-GLUT1 construct was prepared by subcloning the rat GLUT1 cDNA into the pEGFP-C3 plasmid (Clontech) using 5' XhoI and 3' EcoRI restriction sites. The resulting construct contains 9 additional amino acids between the EGFP and GLUT1 (Tyr-Ser-Asp-Leu-Glu-Arg-Ser-Ala-Ala).

Cell culture

Human embryo kidney 293T cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. Chinese hamster ovary cells expressing the human insulin receptor (CHO/IR) were obtained as previously described (Waters S.B., Yamauchi K., and Pessin J.E., "Insulin-stimulated disassociation of the SOS-Grb2 complex." *Mol Cell Biol*, 1995;15:2791-2799.) These cells were maintained in minimal Eagle's medium containing nucleotides plus 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. 3T3L1 preadipocytes were obtained from the American Type Tissue Culture repository and were cultured in DMEM containing 25 mM glucose, 10% calf serum at 37°C in a 8% CO_2 atmosphere. Confluent cultures were induced to differentiate by incubation of the cells with DMEM containing 25 mM glucose, 10% fetal bovine serum, 1 mg/mL insulin, 1 mM dexamethasone, and 0.5 mM isobutyl-1-methylxanthine. After 4 days, the medium was changed to DMEM, 25 mM glucose, 10% fetal bovine serum and 1 mg/mL insulin for an additional 4 days. The medium was then changed to DMEM containing 25 mM glucose and 10% fetal bovine serum. Under these conditions greater than 95% of the cell population morphologically differentiated into adipocytes. The adipocytes were maintained for an additional 4 to 8 days prior to use.

Purification of GST-fusion proteins

Cytoplasmic portions of syntaxin-1A (amino acids 4-264), syntaxin-1B (amino acids 3-263), syntaxin-2 (amino acids 1-265), syntaxin-3 (amino acids 1-262), and syntaxin-4 (amino acids 2-274) were subcloned into pGEX-4T-1 expression vector (Pharmacia). The GST-recombinant proteins were overexpressed in BL21 (DE3) (Stratagene) and bacteria cells were lysed using B-PER-Bacterial Protein Extraction Reagent (Pierce). The GST-fusion proteins were then bound to 50% Glutathione-agarose beads, extensively washed and stored for up to 2 weeks at 4°C.

GST fusion protein precipitation

Cell lysates from HEK293T, CHO/IR or 3T3L1 adipocytes were incubated with either GST alone or with GST-fusion proteins immobilized on glutathione-agarose beads for 1 hour at 4°C. The beads were washed extensively three times with 1 mL HNTG (50 mM HEPES, pH 7.4, 150 mM sodium chloride, 1% Triton X-100, 10% glycerol, and 1 mM EDTA) buffer and then two times with 1 mL distilled water. The retained proteins were then eluted with 50 µL 2X Laemmli sample buffer, heated at 100°C for 5 minutes and separated by SDS-PAGE, and then immunoblotted with the Flag M_2 monoclonal antibody or a sheep polyclonal SYNIP antibody.

Immunoprecipitation and immunoblotting

Whole cell detergent extracts were prepared by detergent solubilization in a NP-40 lysis buffer (25 mM Tris pH 7.4, 1% NP-40, 10% glycerol, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 137 mM NaCl, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, 1 µg/mL pepstatin, 5 µg/mL leupeptin) for 10 minutes at 4°C. Immunoprecipitations were performed by using 4.0 mg of the cell extracts incubated with 8 µg of a syntaxin-4 polyclonal sheep antibody for 2 hours at 4°C. The syntaxin-4 antibody was prepared in sheep using a GST fusion protein expressing the cytoplasmic domain of syntaxin-4 (Olson A.L., Knight J.B., and Pessin J.E., "Syntaxin 4, VAMP2, and/or VAMP3/cellubrevin are functional target membrane and vesicle SNAP receptors for insulin-stimulated GLUT4 translocation in adipocytes." *Mol Cell Biol*, 1997;17:2425-2435.) The samples were then incubated with protein A-Sepharose for 2 hours at 4°C. The resulting immunoprecipitates were then subjected to SDS-polyacrylamide gel electrophoresis and western blotted using the syntaxin-4 polyclonal antibody and the Flag M_2 monoclonal antibody.

Transfection of HEK293T cells, CHO/IR cells and 3T3L1 adipocytes

HEK293T cells were transfected with a mammalian $CaPO_4$ transfection kit (Stratagene). CHO/IR cells were quantitatively transfected by electroporation as previously described (Yamauchi K., Ribon V., Saltiel A.R., and Pessin J.E.,

"Identification of the major SHPTP2-binding protein that is tyrosine-phosphorylated in response to insulin." *J Biol Chem*, 1995;270;17716-17722). Briefly, these cells were mixed with a total of 40 μg of plasmid DNA and electroporated at 340 V and 960 μF. Under these conditions greater than 95% of the surviving cell population express the cDNA of interest. Differentiated 3T3L1 adipocytes were electroporated using a modification of this protocol. The adipocytes were put into suspension by mild trypsinization and electroporated with a total of 600 μg plasmid under low voltage conditions (160 V, 960 μF). The cells were then allowed to adhere to collagen-coated tissue culture dishes for 30-48 hours and the adipocytes were then were serum starved for 2 hours prior to incubation in the absence or presence of 100 nM insulin for 15 minutes at 37°C. Under these conditions, approximately 15% of the electroporated adipocytes survived but of these cells there was greater than a 70% transfection/expression efficiency.

***In situ* β-galactosidase staining**

Differentiated 3T3L1 adipocytes were electroporated with various amounts of plasmid DNA containing the LacZ gene (pcDNA3.1/his/LacZ) as described above, washed with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2.6 mM KH_2PO_4, pH 7.4) and fixed with 2% formaldehyde, 0.2% glutaraldehyde in PBS, pH 7.4 for 10 minutes at room temperature. The cells were then rinsed and incubated with 0.2% 5-bromo-4-chloro-3-indolyl β,D-galactoside reagent (X-Gal) in 10 mM Na_2HPO_4, pH 7.4, 1 mM $MgCl_2$, 150 mM NaCl, 3.3 mM $K_3Fe(CN)_6$, 3.3 mM $K_4Fe(CN)$ for 2 hours at 37°C. The cells were then stored under 70% glycerol and photographed at 100× magnification.

2-Deoxyglucose transport

The electroporated 3T3L1 adipocytes were placed in DMEM containing 25 mM glucose plus 0.5% bovine serum albumin for 2 hours at 37°C. The cells were then washed with KRPH buffer (5 mM Na_2HPO_4, 20 mM HEPES, pH 7.4,

1 mM $MgSO_4$, 1 mM $CaCl_2$, 136 mM NaCl, 4.7 mM KCl, and 1% bovine serum albumin) and either untreated or stimulated with 100 nM insulin for 15 minutes at 37°C. Glucose transport was determined by incubation with 50 µM 2-deoxyglucose containing 0.5 mCi of [^{3}H]2-deoxyglucose in the absence or presence of 10 µM cytochalasin B. The reaction was stopped after 10 minutes by washing the cells 3 times with ice cold PBS. The cells were then solubilized in 1% Triton X-100 at 37°C for 30 minutes and aliquots were subjected to scintillation counting. Protein concentration was determined by the method of Bradford.

Confocal fluorescence microscopy

Differentiated 3T3L1 adipocytes were electroporated as described above with 50 µg of pcDNA3-GLUT4-eGFP and 200 µg of either pcDNA3, SYNIP/WT, SYNIP/NT, or SYNIP/CT. Forty-eight hours after electroporation, the cells were serum starved in DMEM media for 3 hours and incubated with or without 100 nM insulin for 30 minutes. Insulin was removed by two washes with ice cold PBS, fixed with 2% paraformaldehyde (in PBS) for 15 minutes at room temperature, and quenched with 100 mM glycine for 15 minutes at room temperature. Fluorescent cells were visualized by scanning confocal microscopy at the University of Iowa Microscope Facility.

Results

Identification of SYNIP, a multidomain Syntaxin-4 interacting protein

To isolate binding protein(s) that might interact with and regulate the function of syntaxin-4 in insulin-responsive tissues, a yeast two hybrid 3T3L1 adipocyte cDNA library fused to the GAL4 transcription activation domain (Printen J.A., Brady M.J., and Saltiel A.R. "PTG, a protein phosphatase 1-binding protein with a role in glycogen metabolism." *Science*, 1997;275:1475-1478) with the cytoplasmic portion of syntaxin-4 fused to the DNA binding domain of GAL4 as bait (GAL4-Syn4) was screened. Among one million transformants screened, 200 colonies grew on His-Trp-Leu- synthetic medium, of which 102 were positive for β-galactosidase activity when plated on X-Gal containing

medium. Library-derived plasmids were recovered for DNA sequencing and focus was given to one class of cDNAs that induced β-galactosidase activity only when coexpressed with GAL4-Syn4, but not with a fusion protein containing the cytoplasmic domain of syntaxin-3. DNA sequences of the GAL4 fusion junctions of the plasmid inserts encoded for different domains of protein fragments overlapping at the carboxyl terminus. To obtain the upstream 5′ end of the cDNA, 5′-RACE was carried out and an additional 1.2 kb was cloned. The longest 2.6 kb cDNA was obtained by ligating the original 1.4 kb two-hybrid clone with the 1.2 kb 5′-RACE clone. The nucleotide sequence of the SYNIP gene is set forth in Figure 8. Sequence analysis revealed that this cDNA had a single open reading frame which encoded for a 557 amino acid protein with predicted molecular weight of 61 kDa (Fig. 1A). This protein was designated SYNIP for syntaxin-4 interacting protein.

A search of the data bases revealed two mouse EST clones (AA756269 and AA919678) spanning the translation start site of SYNIP. Protein sequence analysis indicated that SYNIP has three specific protein-protein interaction domains: a single PDZ domain at the amino terminus, a pair of tandem coiled-coil domains and a WW domain at the carboxyl terminus (Fig. 1B). In addition, SYNIP contains a potential calcium binding EF-hand motif carboxyl terminal to the predicted PDZ domain and amino terminal to the coiled-coil domains. All these motifs are underlined in the primary amino acid sequences in Figure 1A.

The tissue distribution of SYNIP mRNA was determined using a mouse multiple tissue Northern blot hybridized with a radiolabeled probe containing 1.67 kb of the SYNIP coding sequence (Fig. 1C). A 7.5 kb transcript was predominantly found in skeletal muscle and heart, with substantially lower expression in testis. Two additional transcripts with smaller sizes were also detected, but they did not display a similar restricted tissue distribution pattern. There were no specific SYNIP transcripts in brain, liver, spleen, lung, or kidney tissues. Furthermore, SYNIP mRNA was also detected in rat white and brown adipocytes by Northern blotting (data not shown).

To determine the specificity of SYNIP binding, Flag epitope tag SYNIP constructs were prepared for both full-length SYNIP (SYNIP/WT) and the carboxyl terminal SYNIP domain (SYNIP/CT) encoding for the tandem coiled-coil and WW domains. These constructs were then transfected into HEK293T cells and incubated with either GST alone or GST fusion protein containing the cytoplasmic domains of syntaxin-1A, syntaxin-1B, syntaxin-2, syntaxin-3, and syntaxin-4. *In vitro* binding analysis demonstrated that both SYNIP/WT and SYNIP/CT bound specifically to syntaxin-4 (Fig. 1D, lane 6) but not to the other syntaxin proteins 1A, 1B, 2, and 3 (Fig. 1D, lanes 2-5). Thus, the specific expression of SYNIP in tissues that are enriched in syntaxin-4 and that exclusively display insulin-sensitive glucose transport (muscle and fat), is consistent with a potential role for this protein as a physiologically relevant regulator of GLUT4 translocation.

Insulin disrupts the interaction between SYNIP and syntaxin-4

Previous studies have demonstrated that VAMP2 functions as a GLUT4 vesicle v-SNARE and that the interaction of VAMP2 with syntaxin-4 is necessary for insulin-stimulated GLUT4 translocation to the plasma membrane (Cain C.C., Trimble W.S., and Lienhard G.E., "Members of the VAMP family of synaptic vesicle proteins are components of glucose transporter-containing vesicles from rat adipocytes." *J Biol Chem*, 1992;267:11681-11684; Cheatham B., Volchuk A., Kahn C. R., Wang L., Rhodes C.J., and Klip A., "Insulin-stimulated translocation of GLUT4 glucose transporters requires SNARE-complex proteins." *Proc Natl Acad Sci USA*, 1996;93:15169-15173; Jagadish M.N., Fernandez C.S., Hewish D.R., Macaulay S.L., Gough K.H., Grusovin J., Verkuylen A., Cosgrove L., Alafaci A., Frenkel M.J., and Ward C.W., "Insulin-responsive tissues contain the core complex protein SNAP-25 (synaptosomal-associated protein 25) A and B isoforms in addition to syntaxin-4 and synaptobrevins 1 and 2." *Biochem J*,1996;317:945-954; Martin L.B., Shewan A., Millar C.A., Gould G.W., and James D.E., "Vesicle-associated membrane protein 2 plays a specific role in the insulin-dependent trafficking of the facilitative glucose transporter GLUT4 in 3T3-L1 adipocytes." *J Biol Chem*, 1998;273:1444-1452; Olson A.L., Knight J.B., and Pessin J.E., Syntaxin-4, VAMP2, and/or

VAMP3/cellubrevin are functional target membrane and vesicle SNAP receptors for insulin-stimulated GLUT4 translocation in adipocytes." *Mol Cell Biol*, 1997:17:2425-2435; Tamori Y., Hashiramoto M., Araki S., Kamata Y., Takahashi M., Kozaki S., and Kasuga M., "Cleavage of vesicle-associated membrane protein (VAMP)-2 and cellubrevin on GLUT4-containing vesicles inhibits the translocation of GLUT4 in 3T3-L1 adipocytes." *Biochem Biophys Res Commun*,1996;220:740-745; Volchuk A., Sargeant R., Sumitani S., Liu Z., He L., and Klip A., "Cellubrevin is a resident protein of insulin-sensitive GLUT4 glucose transporter vesicles in 3T3-L1 adipocytes." *J Biol Chem*, 1995;270:8233-8240).

To explore the potential regulation of the SYNIP:syntaxin-4 interaction by insulin, the association of these two proteins was evaluated by co-immunoprecipitation in Chinese hamster ovary cells expressing the human insulin receptor (CHO/IR). Cells were transfected with cDNAs encoding for the Flag epitope-tagged full-length SYNIP (SYNIP/WT), the amino terminal SYNIP domain (SYNIP/NT), and the carboxyl terminal SYNIP domain (SYNIP/CT). Immunoblotting of whole cell detergent extracts demonstrated similar expression of SYNIP/WT and SYNIP/NT, with a slightly greater expression of SYNIP/CT in this particular experiment (Fig. 2A, lanes 1, 3, 5). Insulin stimulation had no significant effect on the expression of these proteins (Fig. 2A, lanes 2, 4, 6). As expected, immunoprecipitation of endogenous syntaxin-4 resulted in the co-immunoprecipitation of SYNIP/WT (Fig. 2B, lane 1). However, following insulin treatment there was a marked reduction in the amount of SYNIP/WT that was co-immunoprecipitated with syntaxin-4 (Fig. 2B, lane 2). In contrast, insulin stimulation had no significant effect on the ability of syntaxin-4 to co-immunoprecipitate the SYNIP/NT or SYNIP/CT proteins (Fig. 2B, lanes 3-6). Interestingly, the interaction of SYNIP/NT with syntaxin 4 *in vivo* was significantly less than that seen with either SYNIP/WT or SYNIP/CT (Fig. 2B), suggesting that the carboxyl terminal region of SYNIP contains the major syntaxin-4 binding domain. In any case, the amount of immunoprecipitated syntaxin-4 can not account for these differences as it was essentially identical under all these conditions (Fig. 2C).

There are several possible mechanisms that could account for the insulin-stimulated dissociation of SYNIP from syntaxin-4. Since there was no apparent

change in SYNIP or syntaxin-4 expression, it seemed most likely that insulin could induce a functional alteration in either SYNIP or syntaxin-4. To explore these possibilities, the ability of GST-syntaxin-4 (GST-Syn4) and GST-SYNIP (GST-SYNIP) fusion proteins to precipitate their corresponding binding partner was examined (Fig. 3). As previously observed, transfection of CHO/IR cells with the SYNIP/WT, SYNIP/NT and SYNIP/CT cDNAs resulted in similar levels of protein expression (Fig. 3A). Incubation of cells with insulin produced a marked reduction in the amount of SYNIP/WT precipitated with GST-Syn4 in cell extracts (Fig. 3B, lanes 1 and 2). However, there was no significant difference in the GST-Syn4 precipitation of either SYNIP/NT or SYNIP/CT in extracts derived from insulin-stimulated cells (Fig. 3B, lanes 3-6). In contrast, incubation of extracts from control and insulin-stimulated cells with GST-SYNIP resulted in the identical amount of syntaxin-4 precipitation (Fig. 3C, lanes 1-4).

To confirm that insulin reduced the binding affinity of SYNIP for syntaxin-4, the binding as a function of GST-Syn4 concentration was examined (Fig. 3D). Immunoblots of whole cell detergent extracts demonstrated equal amounts of expressed SYNIP protein in the control and insulin-stimulated cell extracts (Fig. 3D, lanes 1 and 2). Insulin stimulation resulted in a marked reduction in the amount of SYNIP/WT that was precipitated with 10 and 20 μg of GST-Syn4 compared to the control extracts (Fig. 3D, compare lanes 3 with 4 and lanes 5 with 6). However, the difference between the control and insulin-stimulated cell extracts was diminished with increasing amounts of GST-Syn4 (40 μg), and no significant difference was observed at 80 mg (Fig. 3D, compare lanes 7 with 8 and lanes 9 with 10). The saturation of SYNIP/WT binding was also specific at these concentrations of GST-Syn4 as there was no detectable precipitation of SYNIP/WT by GST alone (data not shown). Thus, these data demonstrate that insulin stimulation results in a specific modification of SYNIP that reduces its ability to associate with syntaxin-4. Furthermore, the decreased binding between SYNIP and syntaxin-4 results from a change in SYNIP binding affinity with no significant alteration in the number of SYNIP or syntaxin-4 binding sites.

Insulin regulates the interaction of SYNIP with syntaxin-4 in 3T3L1 Adipocytes

In contrast to CHO/IR cells, 3T3L1 adipocytes respond to insulin with respect to glucose transport and GLUT4 translocation. It was therefore determined whether the interaction between SYNIP and syntaxin-4 was also sensitive to insulin in these cells. Differentiated 3T3L1 adipocytes were transfected by electroporation with the cDNAs encoding for SYNIP/WT and SYNIP/CT (see Figure 5). Immunoblotting of whole cell lysates demonstrated expression of both SYNIP/WT and SYNIP/CT which was not affected by insulin treatment (Fig. 4A, lanes 1-4). Similar to that observed in CHO/IR cells, incubation of insulin-stimulated cell extracts with GST-Syn4 resulted in a marked decrease in the precipitation of SYNIP/WT but not SYNIP/CT compared to control cell extracts (Fig. 4B, lanes 1-4). These data recapitulate the findings in CHO/IR cells and demonstrate that insulin regulates the interaction between SYNIP and syntaxin-4 in a metabolic insulin-responsive cell type.

SYNIP plays a crucial role in insulin-stimulated glucose transport and GLUT4 translocation

Several studies have also suggested that syntaxin-4 function is necessary for insulin-stimulated GLUT4 vesicle translocation but not GLUT1 vesicle trafficking (Cheatham B., Volchuk A., Kahn C. R., Wang L., Rhodes C.J., and Klip A., "Insulin-stimulated translocation of GLUT4 glucose transporters requires SNARE-complex proteins." *Proc Natl Acad Sci USA*, 1996;93:15169-15173; Olson A.L., Knight J.B., and Pessin J.E., Syntaxin-4, VAMP2, and/or VAMP3/cellubrevin are functional target membrane and vesicle SNAP receptors for insulin-stimulated GLUT4 translocation in adipocytes." *Mol Cell Biol*, 1997:17:2425-2435; Tamori Y., Hashiramoto M., Araki S., Kamata Y., Takahashi M., Kozaki S., and Kasuga M., "Cleavage of vesicle-associated membrane protein (VAMP)-2 and cellubrevin on GLUT4-containing vesicles inhibits the translocation of GLUT4 in 3T3-L1 adipocytes." *Biochem Biophys Res Commun*,1996;220:740-745; Volchuk A., Wang Q., Ewart H.S., Liu Z., He L., Bennett M.K., and Klip A., "Syntaxin 4 in 3T3-L1 adipocytes: regulation by insulin and participation in insulin-dependent glucose transport." *Mol Biol Cell*,

1996;7:1075-1082). However, biochemical analyses of these interactions have been inadequate, since efficient transfection/expression of cDNAs in differentiated 3T3L1 adipocytes is notoriously difficult. To circumvent this issue, a low voltage electroporation method was established for fully differentiated adipocytes which provided for efficient expression using high concentrations of plasmid DNA (Fig. 5A). As a marker for transfection/expression efficiency, differentiated 3T3L1 adipocytes were electroporated (160 V, 960 μF) with various amounts of a cDNA encoding for β-galactosidase (LacZ). Electroporation with the empty vector did not result in any detectable X-Gal staining (Fig. 5A, panel 1). In contrast, electroporation with the LacZ plasmid resulted in a concentration-dependent increase in adipocytes staining positive for β-galactosidase activity (Fig. 5A, panels 2-5). Electroporation with 600 μg of the LacZ expression plasmid routinely results in greater than 70% transfection efficiency with no detectable expression from contaminating fibroblasts.

Having established a reasonable transfection protocol for differentiated 3T3L1 adipocytes, next was examined the effect of SYNIP expression on insulin-stimulated glucose transport (Fig. 5B). Cells electroporated with the empty vector (pcDNA3) remained sensitive to insulin with a 4-fold stimulation of 2-deoxyglucose uptake in these cells. Although expression of SYNIP/WT and SYNIP/NT tended to increase the basal uptake of 2-deoxyglucose, exposure of these cells to insulin resulted in an activation of glucose transport similar to that observed in cells transfected with the empty vector. In contrast, expression of SYNIP/CT slightly inhibited the basal rate of glucose transport, but significantly blunted the insulin-stimulated increase.

3T3L1 adipocytes express both the GLUT1 and GLUT4 glucose transporter isoforms (Calderhead D.M., Kitagawa K., Lienhard G.E., and Gould G.W., "Translocation of the brain-type glucose transporter largely accounts for insulin stimulation of glucose transport in BC3H-1 myocytes." *Biochem J*, 1990;269:597-601; Yang J. and Holman G.D., "Comparison of GLUT4 and GLUT1 subcellular trafficking in basal and insulin-stimulated 3T3-L1 cells." *J Biol Chem*, 1993;268:4600-4603). Although GLUT1 primarily resides on the cell surface in the basal state, it can also undergo insulin-stimulated translocation

to the plasma membrane (Holman G.D., Kozka I.J., Clark A.E., Flower C.J., Saltis J., Habberfield A.D., Simpson I.A., and Cushman S.W., "Cell surface labeling of glucose transporter isoform GLUT4 by bis-mannose photolabel. Correlation with stimulation of glucose transport in rat adipose cells by insulin and phorbol ester." *J Biol Chem*, 1990;265:18172-18179; Piper R.C., Hess L.J., and James D.E., "Differential sorting of two glucose transporters expressed in insulin-sensitive cells." *Am J Physiol*, 1991;260:C570-580; Robinson L.J., Pang S., Harris D.S., Heuser J., and James D.E., "Translocation of the glucose transporter (GLUT4) to the cell surface in permeabilized 3T3-L1 adipocytes: effects of ATP insulin, and GTP gamma S and localization of GLUT4 to clathrin lattices." *J Cell Biol*, 1992;117:1181-1196). Thus, to distinguish the effect of SYNIP expression on GLUT1 and GLUT4 translocation, 3T3L1 adipocytes were transfected with enhanced Green Fluorescent Protein tagged GLUT4 (GLUT4-eGFP) and GLUT1 (eGFP-GLUT1) cDNAs (Fig. 6). In control cells, GLUT4-eGFP was localized to a perinuclear region and to discrete intracellular vesicles throughout the cell interior, but not at the cell surface (Fig. 6A, panel 1). This pattern of GLUT4-eGFP protein expression is identical to that observed for endogenous GLUT4 and co-localizes with another protein marker for the insulin-responsive GLUT4 vesicles, vp165/IRAP (data not shown). Insulin stimulation resulted in a redistribution of the intracellular localized GLUT4-eGFP to the plasma membrane (Fig. 6A, panel 2). These data demonstrate that the expressed GLUT4-eGFP in 3T3L1 adipocytes undergoes the characteristic insulin-stimulated translocation to the plasma membrane, reminiscent of endogenous GLUT4. Consistent with the glucose transport data, expression of SYNIP/WT and SYNIP/NT had no effect on the insulin-stimulated translocation of GLUT4-eGFP (Fig. 6A, panels 2-6). Although expression of SYNIP/CT did not alter the basal distribution of GLUT4-eGFP, there was a near complete inhibition of plasma membrane rim fluorescence (Fig. 6A, panels 7 and 8).

In contrast to GLUT4, a large proportion of GLUT1 is found localized to the plasma membrane in the basal state (Rea S. and James D.E., "Moving GLUT4: the biogenesis and trafficking of GLUT4 storage vesicles." *Diabetes*, 1997;46:1667-1677; Yang J. and Holman G.D., "Comparison of GLUT4 and

GLUT1 subcellular trafficking in basal and insulin-stimulated 3T3-L1 cells." *J Biol Chem*, 1993;268:4600-4603). Similarly, expression of eGFP-GLUT1 also resulted in both plasma membrane and intracellular localization in the basal state (Fig. 6B, panel 1). As expected, transfection with the empty vector had no significant effect on the distribution of eGFP-GLUT1 (Fig. 6B, panel 2). In addition, expression of SYNIP/WT, SYNIP/NT, or SYNIP/CT did not affect either the basal or insulin-stimulated localization of eGFP-GLUT1 (Fig. 6B, panels 2-8). Thus, the inhibition of insulin-stimulated glucose transport activity by SYNIP/CT was specific for GLUT4 translocation, without any significant effect on GLUT1 subcellular distribution.

EXAMPLE 2

Cloning of a cDNA Encoding Human SYNIP

In order to identify hSYNIP, a human sequence database was queried with the nucleotide sequence encoding for mouse SYNIP. A human expressed sequence tag (EST) clone, AA652491, was identified. This EST exhibited 86% homology to the mouse SYNIP sequence.

In order to obtain a full-length human clone, we used the sequence information from the EST to design a polymerase chain reaction (PCR) strategy utilizing Rapid Amplification of cDNA Ends (RACE). Two forward PCR primers, 5′-AGCCCACAAAGGAACAACACCAAGCC-3′ and 5′–GCTCAAGTGTGAAGAGATGATGCC-3′, were designed for 3′ nested PCR RACE and two reverse primers, 5′-GGCATCATCTCTTTCACACTTGAGC-3′ and 5′-GCAAGCAAAACAAGTTTCTGGCAACC-3′ were designed for 5′ nested RACE. Reactions were carried out using a Clontech human fat RACE library.

After completing the 5′ and 3′RACE reactions, the resulting sequences were combined to obtain the full length sequence. To confirm that the 5′RACE and 3′RACE sequences were from the same gene, a 5′ forward primer surrounding the ATG start codon was designed. Using this oligonucleotide, along with a 3′ reverse primer surrounding the stop codon, another PCR reaction was preformed, and a single band was amplified, confirming the identity of the cDNA.

The resulting clone was subject to a final sequence analysis, yielding the complete human SYNIP cDNA sequence.

It is to be understood that the invention is not to be limited to the exact details of operation, or to the exact compounds, compositions, methods, procedures, or embodiments shown and described, as obvious modifications and equivalents will be apparent to one skilled in the art, and the invention is therefore to be limited only by the full scope of the appended claims.